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## Review

# Accepting higher morbidity in exchange for sacrificing fewer animals in studies developing novel infection-control strategies



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## HIGHLIGHTS

- Animal experiments are indispensable proof-of-principle evaluation of new, infection-control strategies.
- Morbidity and mortality must be mimicked in animal infection models in order to better predict human clinical outcome.
- Prevention of death and recurrence must be primary efficacy targets in animal infection models.
- Secondary efficacy targets indirectly relevant for infection-control must not be pursued at the expense of animal lives.
- Exploring mechanisms of action for infection-control strategies must only be done *in vitro*, but not in animal models.

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## ABSTRACT

Preventing bacterial infections from becoming the leading cause of death by the year 2050 requires the development of novel, infection-control strategies, building heavily on biomaterials science, including nanotechnology. Pre-clinical (animal) studies are indispensable for this development. Often, animal infection outcomes bear little relation to human clinical outcome. Here, we review conclusions from pathogen-inoculum dose-finding pilot studies for evaluation of novel infection-control strategies in murine models. Pathogen-inoculum doses are generally preferred that produce the largest differences in quantitative infection outcome parameters between a control and an experimental group, without death or termination of animals due to having reached an inhumane end-point during the study. However, animal death may represent a better end-point for evaluation than large differences in outcome parameters or number of days over which infection persists. The clinical relevance of lower pre-clinical outcomes, such as bioluminescence, colony forming units (CFUs) retrieved or more rapid clearance of infection is unknown, as most animals cure infection without intervention,

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depending on pathogen-species and pathogen-inoculum dose administered. In human clinical practice, patients suffering from infection present to hospital emergency wards, frequently in life-threatening conditions. Animal infection-models should therefore use prevention of death and recurrence of infection as primary efficacy targets to be addressed by novel strategies. To compensate for increased animal morbidity and mortality, animal experiments should solely be conducted for pre-clinical proof of principle and safety. With the advent of sophisticated *in vitro* models, we advocate limiting use of animal models when exploring pathogenesis or infection mechanisms.

## 1. Introduction

Infection is predicted to become the leading cause of death by the year 2050 [1], a prediction which is predominantly due to the development of infections by antimicrobial-resistant pathogens [2]. Preventing this forecast from becoming clinical reality requires increased development of novel infection-control strategies [3], i.e. strategies aimed to treat (therapeutic-mode) or prevent (prophylactic-mode) bacterial infection. Development pathways currently combine drug development and administration with biomaterials science [4] and nanotechnology [3]. Human clinical trials are required as the final step in introducing new drugs to the market [5]. However, human clinical trials are costly [5] and often require enrollment of large numbers of patients in control and experimental groups [6]. Deliberately infecting human volunteers with an antibiotic-resistant pathogen is an ethically unacceptable pathway to conduct human clinical trials, as has been done for instance, in the so-called “Elek-experiment”. The Elek-experiment [7] was first described in 1957 in an era during which the faith in newly discovered penicillin was unlimited [8] and deliberately infecting human volunteers for scientific reasons was therefore considered acceptable. The Elek clinical trial focused on a specifically hard-to-treat infection associated with an implanted biomaterial [9,10]. In a biomaterial-associated infection, bacteria reside in a biofilm-mode of growth, providing pathogen protection against host immune responses and other environmental threats, such as posed by antibiotics. Elek and Conen [7] soaked sutures in a staphylococcal suspension, after which volunteers received three infected sutures in the thigh: two stitches were tied within the thigh, while a third stitch was pulled through the skin and removed. After 24 h, volunteers became very ill and the tied-in sutures had to be removed. The stitches pulled through the skin gave no visible reaction. The study concluded that the presence of an implanted biomaterial enhanced infection, but also that the experiment “led to great difficulty in finding further volunteers” [7].

Since, most development pathways for novel infection-control strategies rely largely on *in vitro* and pre-clinical (animal) experiments. However, animal experiments are costly and becoming more strictly regulated worldwide [11], while societal opposition to animal experiments is growing [12]. Yet, no regulatory agency will approve new antimicrobials without pre-clinical, animal safety data. These constraints have stimulated the development of highly sophisticated *in vitro* methodologies, such as 3D-tissue infection [13] and organ-on-a-chip

models [14,15] and various types of co-culture methods in which multiple key-elements of infection, including bacteria, tissue cells and immune cells, are incorporated [16,17]. Yet, it is unlikely that an *in vitro* experiment will ever completely simulate the complex and dynamic situation witnessed in vertebrate organisms, either animal [10,18] or human. The use of animals in the development and validation of new antimicrobial strategies is substantial, despite the increasing recognition that animal studies have a limited predictive value for human clinical outcome, a consequence that further fuels societal opposition [27–29]. Table 1 (largely taken from Ref. [10]) summarizes a number of possible reasons for the limited predictive value of animal experiments. Bacterial culturing in optimal media is a necessary step preceding inoculation of an animal, but the transition of bacteria from an optimal medium to an *in vivo* environment may have severe consequences on their phenotype, with largely under-studied consequences [10]. Often, human pathogens are selected to infect animals. Although this may seem like a most relevant thing to do, the opposite can also be argued since many human pathogens may not be virulent in animals and possibly *vice versa*. In addition, many infections involve colonization transitions by opportunistic pathogens to an invasive disease over an unpredictable time-span that may well be longer than can be maintained in animal studies. This makes novel prophylactic infection-control strategies more difficult to study in animal experiments than therapeutic ones. Quantification methodology is mostly geared towards quantifiable numerical outcomes amenable to statistical comparison on a continuous-scale, rather than to the binary clinical outcome of treatment: “sick” (due to infection) or “healed”. Although continuous-scale efficacy targets can be defined that bear relation with binary efficacy targets for the treatment of human clinical infections, *in vitro* efficacy targets are generally set too low for human clinical relevance. The consequences of inoculating an animal even with high inoculum doses are much milder than in humans. Animal models intended to support new infection-control strategies for chronically infected wounds seldom deal with wounds that have been demonstrated to be chronically infected but rather apply infected, acutely-inflicted wounds that also cure without intervention. Use of young animals can only be justified for bacterial infections in neonates or young children, but not for elderly frequently suffering from immune compromise and multiple diseases.

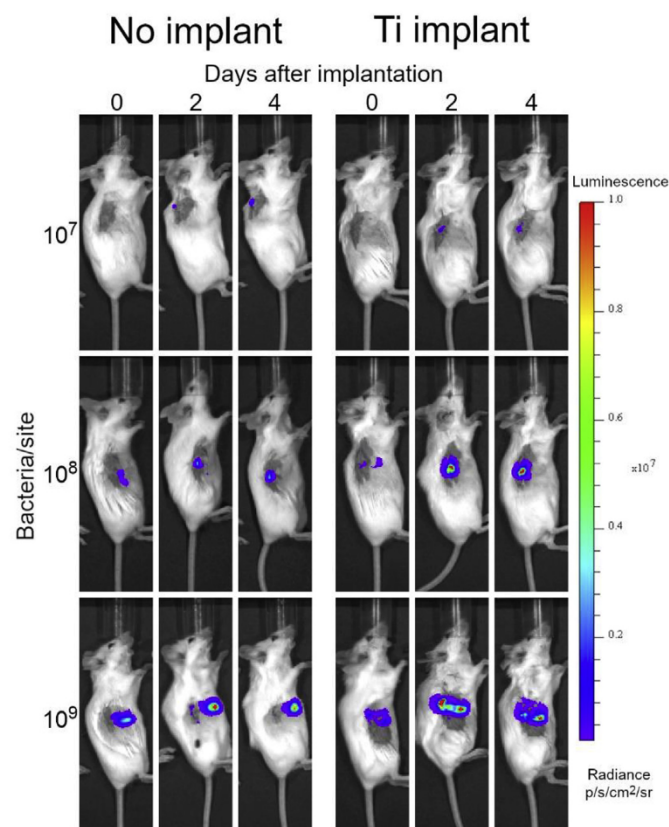
Many of the reasons suggested for the limited predictive value of animal experiments are microbiological in nature and hard to

**Table 1**

Summary of suggested reasons in the literature for the limited predictive value of animal experiments for predicting human clinical outcome in infection-control studies (adapted from Ref. [9]).

Animal study feature	Suggested reasons for poor predictive value for human clinical outcome	Reference
Bacterial inoculum preparation	Bacteria are grown for inocula in optimal, complex media unlike in clinical situations.	[19]
Pathogen selection	Bacterial strain selection can bias results of animal studies.	[20–22]
Quantification methodology	Different methodologies yield different outcomes; colony forming units (CFUs) as the gold standard may not always be reliable due to non-culturable bacteria.	[23–25]
Large inoculum doses	Often enormous, acute bacterial inoculum doses ( $10^6$ – $10^9$ CFU/site) are required for producing reliable infection in animals.	[10]
Subcutaneous infection	Subcutaneous infections are relatively mild compared to life-threatening tissue or organ infections in humans.	[26]
Use of healthy animals	Healthy, young animals are utilized, as opposed to usually elderly human patients, suffering immune compromise and multiple diseases.	[10]
Efficacy targets	Log-3 order (99.9%) in CFU reduction or shorter presence of demonstrable numbers of pathogens are applied in animal models, which do not reflect efficacy targets for the treatment of human clinical infections.	[10]





**Fig. 1.** Examples of IVIS (In Vivo Imaging System) dark box, bioluminescence images of mice, infected with different inoculum doses of bioluminescent *S. aureus* Xen36 taken directly and then at day 2 and 4 after injection of a staphylococcal suspension (20  $\mu$ L) in the absence and presence of an implanted titanium-disc. Staphylococcal injection onto the implanted titanium-disc surface occurred after surgical-closure of the wound.

circumvent, but quantification methodology and efficacy targets in combination with inoculum doses are intimately-related features of animal studies that are within our control. This review aims to provide an alternative perspective for the utility of animal infection models in order to improve their predictive value for human clinical outcome, focusing on study features obviously within our control. In animal infections models, bio-optical imaging of bioluminescent pathogens, sometimes supplemented with more sensitive CFU enumeration from tissue or explanted biomaterials (the present “gold” standard), arguably has become the most commonly applied quantification methodology [30,31]. First, we briefly appraise pathogen-inoculum dose-finding results in animal infection models as presented in the literature. Since published pathogen-inoculum doses are usually posed without thorough description of underlying pilot studies, the appraisal is complemented with results from two typical pathogen-inoculum dose-finding pilot studies in mice involving bio-optical imaging involving biomaterial-associated infection and meningitis. These infections were chosen because both are severe and the human equivalent comes with high morbidity and mortality. Results are subsequently evaluated in view of their relevance to the human clinical outcome. Recommendations are proposed to improve the utility of animal infection models for better translation to clinical practice and importantly, reduce numbers of animals sacrificed in pre-clinical infection studies.

## 2. Appraisal of pathogen-inoculum dose-finding for murine infection models

Murine infection models are most commonly reported for antimicrobial preclinical assessment of novel infection-control strategies.

Pathogen-inoculum dose-finding for murine infection models depends on the bacterial strain used, the quantification methodology preferred and the infection site [27,32–40]. Bioluminescence imaging with genetically modified luminescent-enabled pathogens is nowadays used in many studies. Upon first introduction of bio-optical imaging for studying infection models, bio-optical imaging was advocated as a non-invasive quantification methodology. Its use would reduce the numbers of animals required, because “infection could be longitudinally monitored in one and the same mouse” [39]. Unfortunately, in the meantime we may arguably be able to conclude from published literature that the opposite has likely occurred [32]. The detection limit of bioluminescence imaging is rather low at around  $10^5$  CFU [32]. For this reason, many bioluminescence-based studies are supplemented with traditional CFU enumeration. Moreover, bacterial bioluminescence depends on metabolic activity that decreases after several days [34] and may be affected by antibiotic treatment, particularly at sub-MIC (minimal inhibitory concentration) levels [41]. Pathogen-inoculum dose-finding studies usually comprise a wide dose range from  $10^2$  to  $10^8$  CFU/site. Although  $10^2$  CFU/site has been described to be sufficient to invoke reliable biomaterial-associated infections in murine knee joints for bioluminescence imaging, higher pathogen-inoculum doses between  $10^7$  and  $10^8$  CFU/site are generally applied in other murine models. Pathogen-inoculum doses applied may greatly depend on the bioluminescent pathogen involved since host integration of a bioluminescence construct in a stable plasmid (as e.g. in *Staphylococcus aureus* Xen36) yields higher bioluminescence than direct chromosome integration (as e.g. *S. aureus* Xen29) [33].

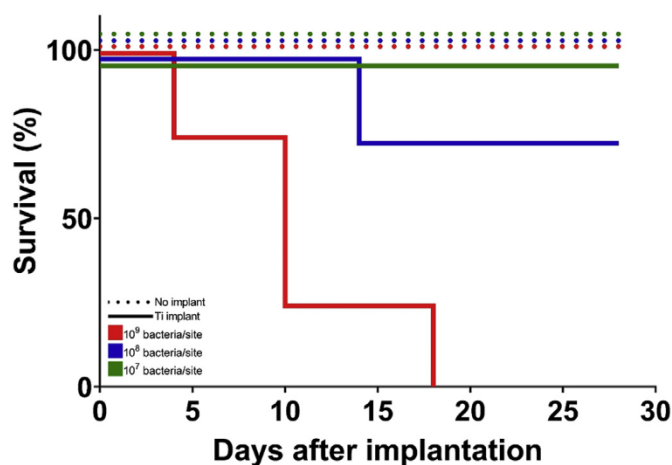
Animal losses are not always reported. A low dose of  $10^4$  CFU/site of *S. aureus* ALC2906 has been reported to cause 100% loss of mice in an evaluation of biomaterial-associated infection in the murine knee [33], while for more frequently applied *S. aureus* Xen29, 100% loss of animals has been reported to occur at a 4 log-unit higher dose of  $10^8$  CFU/site [36]. Also 15–30% loss of animals has been reported for subcutaneous *Pseudomonas aeruginosa* PA14 at doses between  $10^7$  and  $10^8$  CFU/site [34], while a lower dose of  $10^6$  CFU/site caused 100% loss of animals when the pathogen selected was *P. aeruginosa* Xen5 [36].

Since pathogen-inoculum dose-finding pilot studies are not always described in sufficient detail to understand the precise rationale for deciding on a given pathogen-inoculum dose in further larger-powered studies, we report in addition to the studies cited above, on results of two (unpublished) pathogen-inoculum dose-finding pilot studies in mice (see [Supporting Information](#) for experimental details) involving different types of infection.

One dose-finding pilot study was performed as part of a larger-powered animal infection-control experiment, in which antimicrobial effects of a DNaseI coating [42] on peri-operative infections associated with implanted biomaterials were evaluated. This dose-finding pilot study *de facto* represents an animal analogue of the Elek-experiment in humans, but evaluating infection associated with implanted titanium-discs based on bio-optical imaging. Beyond the pilot study presented here, results of the larger-powered animal experiment were considered inconclusive and never published, presumably because improper decisions were made based on the dose-finding pilot study.

**Fig. 1** summarizes examples of bioluminescence images of mice in the different groups of animals without and with an implanted titanium-disc in the absence or presence of different pathogen-inoculum doses of bio-luminescent *S. aureus* Xen36, taken during the initial days after disc implantation and pathogen inoculation of the animals. Clearly, an inoculum dose of  $10^7$  staphylococcal CFU/site constituted too low a dose to image *in situ* growth of bioluminescent *S. aureus* Xen36. More substantial doses of  $10^8$  or  $10^9$  staphylococcal CFU/site yielded clear images of the progression of infection over time, while also showing that the implanted titanium-discs exacerbated the infection, especially in mice infected with  $10^9$  staphylococcal CFU/site.

**Fig. 2** presents survival curves for the different groups of mice. All mice without an implanted titanium-disc survived the experimental



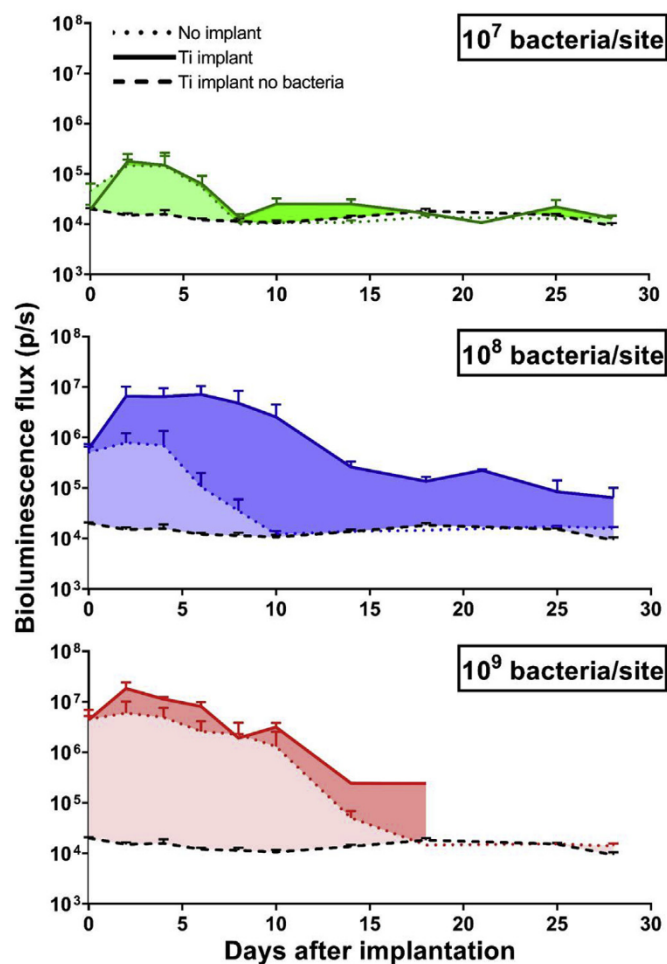
**Fig. 2.** Kaplan-Meier survival curves for mice inoculated with different doses of bioluminescent *S. aureus* Xen36 as a function of time after infection in the absence and presence of an implanted titanium-disc. Each group comprised four mice. Mice were terminated when their condition had reached a pre-defined, humane endpoint, or completion of the study at the designated end of the experiment (day 28).

period without any signs of infection or other adverse side-effects, regardless of pathogen-inoculum dose. However, mice with an implanted titanium-disc and inoculated with the highest dose of  $10^9$  staphylococcal CFU/site presented with purulent wounds near the implant site, consistent with the bioluminescence images in Fig. 1. This infection caused loss of one animal at day 3 and eventual loss of all animals at day 18 after pathogen inoculation. Inoculation with a dose of  $10^8$  staphylococcal CFU/site led to the loss of 1 animal at day 14 in the titanium-implant group, while all animals with an implanted titanium-disc injected with an inoculation dose of  $10^7$  staphylococcal CFU/site survived.

Fig. 3 shows that the bioluminescence flux arising from mice inoculated with a dose of  $10^7$  staphylococcal CFU/site was only slightly higher than from mice inoculated with sterile saline during the first six days after implantation and inoculation. Differences in bioluminescence from mice inoculated in both absence and presence of implanted titanium-discs were negligible. When inoculated with dose of  $10^8$  staphylococcal CFU/site, mice readily cleared pathogen inoculation in absence of an implanted titanium-disc to saline-control levels within 10 days. In the presence of implanted titanium-discs, however, bioluminescence remained well above control levels until the end of the study at day 28. Yet the three surviving animals out of the group of four, exhibited no clinical symptoms of infection. Natural clearance of infection according to bioluminescence output by the mouse immune system in the absence of an implanted titanium-disc at a dose of  $10^9$  staphylococcal CFU/site to saline-control levels occurred slower than at lower doses but still occurred within 18 days after implantation and inoculation and without loss of animals (compare Fig. 2). However, at the highest inoculum dose of  $10^9$  staphylococcal CFU/site and in the presence of an implanted titanium-disc, bioluminescence was only marginally higher in the titanium-implant group than in the group of mice without an implant. However, infection-associated complications necessitated termination of this experimental group at day 18 (see also Fig. 2). Based on this pathogen-inoculum dose-finding pilot study, the dose of  $10^7$  staphylococcal CFU/site was considered too low for reliable infection-control evaluation. The high loss of animals/implants at the highest dose of  $10^9$  staphylococcal CFU/site was considered experimentally unacceptable for various reasons, such as an unpredictable number of surviving mice at end-point and inhumane animal morbidity arising from infection, requiring termination of the animal. Summarizing, we concluded, similar to virtually all pathogen-inoculum dose-finding pilot studies for infection-control evaluation in mice, that an inoculum dose of  $10^7$

staphylococcal CFU/site was too low to yield any useful outcome parameter, while a dose of  $10^9$  staphylococcal CFU/site yielded too many losses for appropriate powering of the study. Accordingly, the decision was made to apply an intermediate pathogen-inoculation dose of  $10^8$  staphylococcal CFU/site, which in a larger-powered murine study presumably led to inconclusive results.

A second dose-finding study (see also Supporting Information for experimental details), was carried out as part of a larger-powered animal infection-control experiment evaluating a new antimicrobial for the control of meningitis. Bacterial meningitis is an extremely serious infection of the brain. *Escherichia coli* is one of the Gram-negative pathogens causing meningitis, in particular during the neonatal period where it is the second most common causative agent [43]. Rapid diagnosis is paramount in conjunction with efficacious antibiotic therapy, otherwise morbidity and often mortality is inevitable within days after the onset of the first symptoms [44]. All mice, regardless of the *E. coli*-inoculum dose injected into the brain, awoke within 1–2 h after inoculation and anesthesia without showing any signs of morbidity (see Table 2) or other signs of distress. At the lowest pathogen-inoculum dose of  $2.5 \times 10^2$  *E. coli* CFU/site, mice demonstrated no signs of morbidity, while at a higher dose of  $2.5 \times 10^3$  *E. coli* CFU/site signs of morbidity disappeared over the course of 48 h. Similarly, at a dose of



**Fig. 3.** Bioluminescence fluxes in mice, inoculated with different doses of bioluminescent *S. aureus* Xen36 as a function of time after inoculation in the absence and presence of an implanted titanium-disc. The differently colored, shaded areas indicate the effects of inoculation in absence of an implanted titanium-disc with respect to the saline-control (no injected staphylococci; light-shaded), while the dark-shaded areas represent the effects of an implanted titanium-disc on infection. Error bars indicate the standard error of the mean over the number of surviving mice in each group (see Fig. 2).



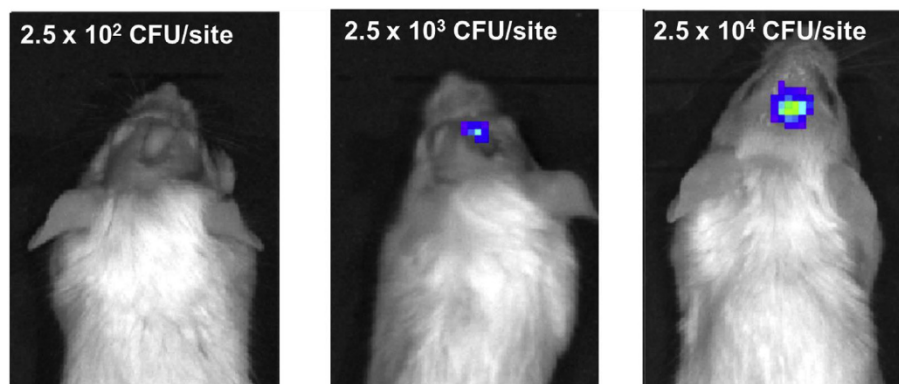
**Table 2**Signs of morbidity and mortality in different groups of six mice, with different *E. coli*-inoculum doses injected (2.5  $\mu$ L) in the brain.

Hours after inoculation	$2.5 \times 10^2$ CFU/site	$2.5 \times 10^3$ CFU/site	$2.5 \times 10^4$ CFU/site	$2.5 \times 10^5$ CFU/site
Death <sup>a</sup>				
12	– <sup>a</sup>	–	–	3/6 <sup>b</sup>
24	–	–	–	5/6
48	–	–	–	6/6
Lethargy <sup>c</sup>				
12	+	+	+	+
24	+	+	+	+
48	–	+	+	f
Not Eating <sup>c</sup>				
12	+	+	+	+
24	–	–	+	+
48	–	–	–	f
Scarcely Drinking <sup>c</sup>				
12	+	+	+	+
24	–	–	+	+
48	–	–	–	f
Reduced Movement/Leaning over to One Side of the Body <sup>c</sup>				
12	+	+	+	+
24	–	–	+	+
48	–	–	+	f
Feverish <sup>d</sup>				
12	–	+++	+++	+++
24	–	++	+++	+++
48	–	+	+++	f
Loss of Body Weight <sup>e</sup>				
12	+	++	+++	+++
24	+	++	+++	+++
48	+	++	+++	f
Photophobic <sup>c</sup>				
12	–	–	+	+
24	–	–	+	+
48	–	–	+	f
Abnormal Behaviour <sup>c</sup>				
12	+	+	+	+
24	+	+	+	+
48	–	+	+	f

<sup>a</sup> Indicates absence of the described symptom.<sup>b</sup> Number of dead mice in a group of six.<sup>c</sup> Symptoms were displayed by all mice in a group.<sup>d</sup> +++: temperature above 38.5 °C, ++: temperature between 38 °C and 38.5 °C, +: temperature between 37.5 and 38 °C, -: temperature less than 37.5 °C.<sup>e</sup> +++: weight loss more than 2.5 g, ++: weight loss between 2.5 and 1.5 g, +: weight loss less than 1.5 g.<sup>f</sup> No surviving mice.

$2.5 \times 10^4$  *E. coli* CFU/site, mice began to eat and drink again after 48 h, which may have alluded the onset of complete disappearance of morbidity and full clearance of infection. Only the highest dose of  $2.5 \times 10^5$  *E. coli* CFU/site, persistent morbidity signs were observed in conjunction with mortality in all mice, which is the typical course of meningitis in humans when left untreated. Bioluminescence imaging (Fig. 4) revealed absence of bioluminescence at the lowest *E. coli*

inoculum dose, but with noticeable bioluminescence in mice inoculated with higher doses. Mice injected with the highest pathogen dose showed the highest bioluminescence. Contrary to the decision taken in the biomaterial-associated infection pilot study, it was here decided to apply the highest pathogen-inoculation dose of  $2.5 \times 10^5$  *E. coli* CFU/site for further studies, because the course of infection then proceeded comparably with human clinical symptoms of untreated meningitis.



**Fig. 4.** Examples of IVIS dark box, bioluminescence images of mice, infected in the brain with different inoculum doses (2.5  $\mu$ L) of bioluminescent *E. coli* Xen14 taken 48 h after brain-injection. Mice injected with a dose of  $2.5 \times 10^5$  *E. coli* CFU/site died prior to bioluminescence imaging.

### 3. Discussion

Bacterial bioluminescence in mice decreased over time in the absence or presence of an implanted titanium-disc after pathogen inoculation (Fig. 3), exactly following expectations for biomaterial-associated infection [44]. This pathogen-inoculum dose-finding pilot study can be considered as the complete murine analogue of the Elek-experiment in humans [7], now carried out with modern evaluation methods. However, the magnitude of bacterial bioluminescence decreases over time, depended heavily on pathogen-inoculum dose, similar as in the meningitis dose-finding pilot study. Virtually all decisions on pathogen-inoculum doses follow the same pattern of considerations in murine and other animal infection models. Unfortunately, pathogen-inoculum dose-finding pilot studies seldom report the considerations underlying any decision for selecting a specific pathogen-inoculum dose in detail. Yet, the choice for a particular pathogen-inoculum dose may critically affect the predictive value of a pre-clinical animal experiment for human clinical outcome in pursuing larger-powered studies. Below, we debate the question of whether we, and many of our peers, used the correct arguments to decide for intermediate doses of around  $10^8$  CFU/site, as in our dose-finding pilot study on peri-operative infections associated with implanted biomaterials. An alternative and more unusual decision was taken in our study on meningitis, in which a high, lethal dose of  $2.5 \times 10^5$  *E. coli* CFU/site was decided upon, causing death of all mice within 48 h. Arguments and counter-arguments for both decisions are presented below:

#### 3.1. Intermediate pathogen-inoculum doses should be used that avoid animal death

The intermediate staphylococcal-inoculum dose in the biomaterial-associated infection model left us a broad experimental window to evaluate possible efficacy of a new infection-control coating, yielding large bioluminescence differences between control and infected groups. Important for publication purposes, loss of animals during the experimental period was expected to be small and inhumane, infection-associated morbidity of animals to be minimal. Ethical and practical aspects common to many experimental designs were prospectively satisfied. A lethally high *E. coli*-inoculum dose as preferred in the meningitis model, does not allow all this and should not be chosen.

#### 3.2. High, lethal pathogen-inoculum doses should be considered preferable

Animal experiments should be directly relevant for the human clinical situation and if they do not meet this condition, they should not be allowed or performed. Humans seek medical intervention when they feel ill. In severe cases for which our research endeavours seek to provide help, infection causes people to become lethargic, they do not eat, scarcely drink, hardly move and develop fever, all of which influence the body's ability to fight the infection. Commonly, a bacterial infection causes drainage, acute organ failure or necessitates immediate life-saving intravenous antibiotic administration or surgery on a heavily suffering patient. If mis-diagnosed or wrongly treated, patients die within days from serious infections, like meningitis [45] or a biomaterial-associated infection like endocarditis, which can arise from a simple pacemaker replacement [46]. A relevant animal experiment should mimic this worst case, clinical courses, including death. The number of annual deaths worldwide due to antimicrobial resistant infection amounts currently around 700,000 [1], and is expected to rise to 10 million by the year 2050 [47] when severe infection may have become the clinical norm. Current research and associated animal use, should be primarily aimed towards preventing just this from becoming true.

The ultimate objective of clinical intervention is to prevent death and recurrence of infection. This rationale would support the choice for

the most aggressive model and highest possible pathogen-inoculation dose for both studies discussed above. Prevention of animal death and recurrence of infection as primary efficacy targets would yield a more robust study with higher translational relevance when compared to aiming for statistically significant reductions in bioluminescence, CFUs or number of days over which infection is present, as most current studies report.

Taking prevention of death as a critical, primary efficacy target requires accepting what might be considered as high levels of animal morbidity and mortality and undoubtedly invoke the highest degree of scrutiny by animal welfare review bodies. However, animal morbidity can be alleviated using pain medication, which will actually add to the resemblance between infection in test animals and humans and therefore to the predictive value of the animal experiment. After all, no expert knows the clinical relevance of a reduced bioluminescence, less CFUs or more rapid clearance of infection, while most animals are also able to clear intermediate inoculum dose infections without intervention. Infection models in animals are seldom designed to allow morbidity and to have death as an end-point to be prevented by a novel treatment. Yet, it can be argued that this much more closely resembles the human situation of a seriously ill person seeking medical care than an animal that is infected to a level that is not morbid, self-clearing and observably hardly bothersome.

As another primary efficacy target, in cases where a new strategy prevents animal death, animals should no longer be sacrificed at the pre-designated end of an experimental period, but instead be followed longitudinally over time to evaluate possible recurrence of infection. Recurrence is a troublesome complication of human clinical infection treatment. Recurrence arises from incomplete eradication of infectious biofilms and infecting pathogens seeking shelter in mammalian cells against antibiotic treatment [48,49]. Prevention of recurrence as a second primary efficacy target in evaluating novel infection-control strategies will likely also add to the predictive value of animal experiments for human clinical outcome, but may go at the expense of e.g. other end-point evaluations such as histology or bacterial CFUs in tissue. In the authors opinion, opting for 1) lethally high pathogen-inoculum doses with death and 2) recurrence of infection as preventable primary efficacy targets, is preferable, simply because death, morbidity and recurrence are unequivocally associated with the human disease symptoms of infection.

Turning the emphasis of infection-control studies to death and recurrence as primary efficacy targets, reduces the impact of quantification methodology (see Table 1) on the final conclusions of a study. Yet, other secondary efficacy targets such as reduced bioluminescence, faster clearance of infection in animal models or end-point bacterial CFU reduction in tissue and histology, may remain useful to improve our understanding of pathogenesis and infection mechanisms, as well as of healing processes occurring upon application of novel infection-control strategies. However, these should only be pursued without additional inconvenience or costs of animal lives. In this respect, bio-optical imaging remains a good assay, because it is non-invasive and allows monitoring the course of infection over time in one and the same animal. However, as a drawback of bio-optical imaging, it should be kept in mind that bio-optical imaging limits pathogen selection (see Table 1) due to the relatively few bioluminescent strains available.

The above suggestion to base infection-control studies in animals primarily on death as an endpoint and recurrence of infection, requires serious diligence to address the increased morbidity. It is clear that to date, that prior to starting human clinical trials, animal experiments are still required to investigate new, infection-control strategies for the occurrence of possible adverse effects using vertebrate animals. Unforeseen problems upon application of new, infection-control strategies in humans, as in the 1957 Elek-experiment [7], must be minimized. Few would argue to move novel infection-control strategies to human clinical trials without animal de-risking. Nonetheless, animal experiments conducted solely upon demands of peer reviewers and



editors of high impact journals with the mere aim to get papers published, should be prohibited. The idea that good, well-founded infection research must always, necessarily involve animal studies is presumed too often and animal studies have become the routine default position for infection research and development without proper justification. Also, when the work has little prospects of downward clinical translation (i.e. because it represents far too complicated methodology, impossible to produce cost-effectively, cannot be sterilized, or lack of commercial interest or funding), animal experiments should not be conducted. We believe that the amount of animal use for demonstration of mechanisms is excessive, often experimentally unjustified and largely without merit or impacting conclusions. Ample, highly sophisticated *in vitro* models are available nowadays [13–17] to establish benefit over a clinical standard and provide clues and guidance to elucidate mechanisms of action of new antimicrobials, warranting scientific publication in the absence of pre-clinical animal evaluation. Animal experiments should only be employed once *in vitro* research has clearly demonstrated benefit over the clinical standard of treatment for a particular type of infection and not for demonstration of mechanisms of action. Moreover, it seems trivial to this authors consortium, not to dedicate much concern, significant research resources, and animal lives on self-resolving infections or those curable with current therapies.

In conclusion, this consortium suggests the use of higher animal morbidity models in exchange for sacrificing fewer animals in the development of novel infection-control strategies in order to perform animal assessment of novel strategies with a greater predictive value for human clinical outcome. Specifically, our suggestions involve:

1. acceptance of animal death and recurrence of infection as primary efficacy targets,
2. considering other infection quantification methodologies such as bio-optical imaging or CFU analysis only as secondary efficacy targets, provided this can be accomplished without additional inconvenience or costs of animal lives,
3. only allowing animal evaluation of novel infection-control strategies when *in vitro* experiments have indicated superiority above the clinical standard of treatment,
4. restricting animal experiments to elucidate mechanisms of action of a new antimicrobial when effective *in vitro* models are available.

These suggestions are put forward with the best intentions towards making animal experiments more useful for predicting human clinical outcome and translating novel infection-control strategies to clinical use. This will create better de-risking and may help in accelerated translation of new infection-control strategies to clinical use. Furthermore, the authors intend to curb unnecessary studies resulting in preventable animal usage for the sake of science only. We will do our utmost best to perform research that meets the above suggestions, in which we sincerely believe. Nevertheless, our scientific curiosity drives us. May our scientific curiosity kill neither the cat nor the mouse and save human lives.

#### 4. Transparency declarations

H.J. Busscher is also director of a consulting company, SASA BV (GN Schutterlaan 4, 9797 PC Thesinge, The Netherlands). T.G. van Kooten is the formally-appointed responsible scientist for animal experiments involving biomaterial-associated infection models within UMCG, which includes writing proposals for the Dutch CCD (Centrale Commissie Dierproeven), discussing protocols with the Animal Welfare Body and deciding on animal termination. T.P. Schaer is a voting member on the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) board. The authors declare no potential conflicts of interest with respect to authorship and/or publication of this article. Opinions and assertions contained herein are those of the authors and are not construed as necessarily representing views of any funding

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#### Appendix A. Supplementary data

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#### References

- [1] G. Humphreys, F. Fleck, United Nations meeting on antimicrobial resistance, Bull. World Health Organ. 94 (2016) 638–639.
- [2] O. Ciofu, T. Tolker-Nielsen, Tolerance and resistance of *Pseudomonas aeruginosa* biofilms to antimicrobial agents – how *P. aeruginosa* can escape antibiotics, Front. Microbiol. 10 (2019) 913.
- [3] Y. Liu, et al., Nanotechnology-based antimicrobials and delivery systems for bio-film-infection control, Chem. Soc. Rev. 48 (2019) 428–446.
- [4] Y. Luan, et al., Bacterial interactions with nanostructured surfaces, Curr. Opin. Colloid Interface Sci. 38 (2018) 170–189.
- [5] H.J. Busscher, et al., Biomaterials-associated infection: locating the finish line in the race for the surface, Sci. Transl. Med. 4 (2012) 153rv10.
- [6] R.O. Darouiche, Antimicrobial approaches for preventing infections associated with surgical implants, Clin. Infect. Dis. 36 (2003) 1284–1289.
- [7] S.D. Elek, P.E. Conen, The virulence of *Staphylococcus pyogenes* for man – a study of the problems of wound infection, Br. J. Exp. Pathol. 38 (1957) 573–586.
- [8] A. Fleming, On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of *B. influenza*, Br. J. Exp. Pathol. 10 (1929) 226–236.
- [9] R.O. Darouiche, Treatment of infections associated with surgical implants, N. Engl. J. Med. 350 (2004) 1422–1429.
- [10] T.F. Moriarty, D.W. Grainger, R.G. Richards, Challenges in linking preclinical antimicrobial research strategies with clinical outcomes for device-associated infections, Eur. Cells Mater. 28 (2014) 112–128.
- [11] T.F. Moriarty, et al., Recommendations for design and conduct of preclinical *in vivo* studies of orthopedic device-related infection, J. Orthop. Res. 37 (2019) 271–287.
- [12] D. Campbell, Public opposition to animal testing grows, The Guardian October 19 (2012).
- [13] X. Ren, H.C. van der Mei, Y. Ren, H.J. Busscher, Keratinocytes protect soft-tissue integration of dental implant materials against bacterial challenges in a 3D-tissue infection model, Acta Biomater. 96 (2019) 237–246, <https://doi.org/10.1016/j.actbio.2019.07.015>.
- [14] H.J. Kim, et al., Human gut-on-a-chip inhabited by microbial flora that experiences intestinal peristalsis-like motions and flow, Lab Chip 12 (2012) 2165–2174.
- [15] T.D. Brown, et al., A microfluidic model of human brain (μHuB) for assessment of blood brain barrier, Bioeng. Transl. Med. 4 (2019) e10126.
- [16] G. Subbiahdoss, et al., *In vitro* interactions between bacteria, osteoblast-like cells and macrophages in the pathogenesis of biomaterial-associated infections, PLoS One 6 (2011) e24827.
- [17] P.P.S. Balraadsing, et al., Poly(trimethylene carbonate) and poly(D,L-lactic acid) modify human dendritic cell responses to staphylococci but do not affect Th1 and Th2 cell development, Eur. Cells Mater. 35 (2018) 103–116.
- [18] R.T. Rozenbaum, et al., Antimicrobial synergy of monolaurin lipid nanocapsules with adsorbed antimicrobial peptides against *Staphylococcus aureus* biofilms *in vitro* is absent *in vivo*, J. Control. Release 293 (2019) 73–83.
- [19] R.C. Massey, et al., Functional blocking of *Staphylococcus aureus* adhesins following growth *in ex vivo* media, Infect. Immun. 70 (2002) 5339–5345.
- [20] D. Campoccia, et al., The selection of appropriate bacterial strains in preclinical evaluation of infection resistant biomaterials, Int. J. Artif. Organs 31 (2008) 841–847.
- [21] K.J. Hendricks, et al., Synergy between *Staphylococcus aureus* and *Pseudomonas aeruginosa* in a rat model of complex orthopaedic wounds, J. Bone Joint Surg. Am 83-A (2001) 855–861.
- [22] A. Korgaonkar, U. Trivedi, K.P. Rumbaugh, M. Whiteley, Community surveillance enhances *Pseudomonas aeruginosa* virulence during polymicrobial infection, Proc. Natl. Acad. Sci. U.S.A. 110 (2013) 1059–1064.
- [23] J.D. Oliver, Recent findings on the viable but nonculturable state in pathogenic bacteria, FEMS Microbiol. Rev. 34 (2010) 415–425.
- [24] M. Palmer, W. Costerton, J. Sewecke, D. Altman, Molecular techniques to detect biofilm bacteria in long bone nonunion: a case report, Clin. Orthop. Relat. Res. 469 (2011) 3037–3042.
- [25] T.S. Howe, G. Erlich, J.S. Koh, A.C. Ng, W. Costerton, A case of an atypical femoral fracture associated with bacterial biofilm – pathogen or bystander? Osteoporos. Int.



- 24 (2013) 1765–1766.
- [26] T. Dai, G.B. Kharkwal, M. Tanaka, Y.-Y. Huang, V.J. Bil de Arce, M.R. Hamblin, Animal models of external traumatic wound infections, *Virulence* 2 (2011) 296–315.
- [27] D.H. Veening-Griffioen, et al., Are some animal models more equal than others? A case study on the translational value of animal models of efficacy for Alzheimer's disease, *Eur. J. Pharmacol.* 859 (2019) 172524.
- [28] A. Akhtar, The flaws and human harms of animal experimentation, *Camb. Q. Healthc. Ethics* 24 (2015) 407–419, <https://doi.org/10.1017/S0963180115000079>.
- [29] L.A. Colby, L.E. Quenee, L.A. Zitzow, Considerations for infectious disease research studies using animals, *Comp. Med.* 67 (2017) 222–231.
- [30] J. Sjollem, et al., The potential for bio-optical imaging of biomaterial-associated infection in vivo, *Biomaterials* 31 (2010) 1984–1995.
- [31] K.P. Francis, et al., Monitoring bioluminescent *Staphylococcus aureus* infections in living mice using a novel luxABCDE construct, *Infect. Immun.* 68 (2000) 3594–3600.
- [32] N.A. Kuklin, et al., Real-time monitoring of bacterial infection in vivo: development of bioluminescent staphylococcal foreign-body and deep-thigh-wound mouse infection models, *Antimicrob. Agents Chemother.* 47 (2003) 2740–2748.
- [33] J.R. Pribaz, et al., Mouse model of chronic post-arthroplasty infection: noninvasive in vivo bioluminescence imaging to monitor bacterial burden for long-term study, *J. Orthop. Res.* 30 (2012) 335–340.
- [34] D. Pletzer, H. Wolfmeier, M. Bains, R.E.W. Hancock, Synthetic peptides to target stringent response-controlled virulence in a *Pseudomonas aeruginosa* murine cutaneous infection model, *Front. Microbiol.* 8 (2017) 1867.
- [35] N. Dinjaski, et al., Near-infrared fluorescence imaging as an alternative to bioluminescent bacteria to monitor biomaterial-associated infections, *Acta Biomater.* 10 (2014) 2935–2944.
- [36] J.L. Kadurugamuwa, et al., Direct continuous method for monitoring biofilm infection in a mouse model, *Infect. Immun.* 71 (2003) 882–890.
- [37] D. Pletzer, S.C. Mansour, K. Wuerth, N. Rahanjam, R.E.W. Hancock, New mouse model for chronic infections by Gram-negative bacteria enabling the study of anti-infective efficacy and host-microbe interactions, *mBio* 8 (2017) e00140-17.
- [38] G. Fila, et al., Murine model imitating chronic wound infections for evaluation of antimicrobial photodynamic therapy efficacy, *Front. Microbiol.* 7 (2016) 1258.
- [39] J.M. Thompson, L.S. Miller, Preclinical optical imaging to study pathogenesis, novel therapeutics and diagnostics against orthopaedic infection, *J. Orthop. Res.* (2019), <https://doi.org/10.1002/jor.24428>.
- [40] K.D. Walton, A. Lord, L.V. Kendall, S.W. Dow, Comparison of 3 real-time, quantitative murine models of staphylococcal biofilm infection by using in vivo bioluminescent imaging, *Comp. Med.* 64 (2014) 25–33.
- [41] S. Daghighi, et al., Influence of antibiotic pressure on bacterial bioluminescence, with emphasis of *Staphylococcus aureus*, *Int. J. Antimicrob. Agents* 46 (2015) 713–717.
- [42] J.J.T.M. Swartjes, et al., A functional DNase I coating to prevent adhesion of bacteria and the formation of biofilm, *Adv. Funct. Mater.* 23 (2013) 2843–2849.
- [43] K.S. Kim, Human meningitis-associated *Escherichia coli*, *EcoSal Plus* 7 (2016) 1–15, <https://doi.org/10.1128/ecosalplus.ESP-0015-2015>.
- [44] A.F. Engelsman, et al., Real time noninvasive monitoring of contaminating bacteria in a soft tissue implant infection model, *J. Biomed. Mater. Res. B Appl. Biomater.* 88B (2009) 123–129.
- [45] R. Hasbun, Update and advances in community acquired bacterial meningitis, *Curr. Opin. Infect. Dis.* 32 (2019) 233–238.
- [46] D.W. Grainger, et al., Critical factors in the translation of improved antimicrobial strategies for medical implants and devices, *Biomaterials* 34 (2019) 9237–9243.
- [47] J. O'Neill, Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations. Review on Antimicrobial Resistance. Creative Commons Attribution 4.0, International Public Licence, 2014.
- [48] J.J. Boelens, et al., Biomaterial-associated persistence of *Staphylococcus epidermidis* in pericatheter macrophages, *J. Infect. Dis.* 181 (2000) 1337–1349.
- [49] R.K. Ernst, T. Guina, S.I. Miller, How intracellular bacteria survive: surface modifications that promote resistance to host innate immune responses, *J. Infect. Dis.* 179 (1999) S326–S330.